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**PATENT APPLICATION**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**



In re application of

Docket No: A8873

RAVI V.J. CHARI

Appln. No.: 09/671,995

Group Art Unit: 1643

Confirmation No.: 2588

Examiner: K.A. Canella

Filed: September 29, 2000

For: COMPOSITIONS AND METHODS FOR TREATING CANCER USING  
IMMUNOCONJUGATES AND CHEMOTHERAPEUTIC AGENTS

**DECLARATION UNDER 37 C.F.R. § 1.132**

Mail Stop Amendment  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

I, Walter A. Blättler, hereby declare and state:

THAT I am a citizen of Switzerland;

THAT I have received the degree of Ph.D. in Chemistry from Swiss Federal Institute of  
Technology;

THAT I have been employed by Immunogen, Inc. since 1987, where I currently hold the  
position of Executive Vice President, Science & Technology.

My Curriculum Vitae is attached to this Declaration.

This Declaration is being provided to the Patent Office to further illustrate and explain  
the unexpected results that are disclosed in U.S. Application No. 09/671,995, as these results  
would be understood by one of ordinary skill in the art.

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First, I wish to provide some comments on synergistic and antagonistic drug combinations.

Although most chemotherapeutic agents have been approved based on their activity as single agents, generally these are not sufficiently active to cause long term disease remissions. Thus, chemotherapeutic agents are often used in combination with the goal of obtaining increased activity. Typically, cytotoxic drugs that have different mechanisms of killing are combined. Such drugs have different targets in the cancer cells and are called mutually exclusive drugs.

Mutually exclusive drugs either behave in an additive, synergistic, or antagonistic manner (Chou and Talalay, *Adv. Enzyme Regul.* 1984, **22**:27-55, a copy of which is attached to this Declaration).

Preclinically, the effect of a combination of cytotoxic drugs can be studied *in vitro* on cell lines or *in vivo* with different tumor models. In such experimental systems most drug combinations show an additive effect. In some instances however, the combinations show less or more than an additive effect. These combinations are called antagonistic or synergistic, respectively. Antagonistic or synergistic effects are unpredictable, and are unexpected experimental findings.

Application No. 09/671,995 discloses synergistic drug combinations with *in vivo* tumor models. Specifically, the drug combinations are an immunoconjugate containing an anti-mitotic agent (a maytansinoid) and a cytotoxic drug from four different categories: taxanes and other

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drugs with a taxane mechanism (pages 2 and 25), platinum compounds (pages 2 and 25), epipodophyllotoxins (etoposide; page 2), and camptothecin compounds (pages 2 and 27).

This declaration provides further experimental evidence demonstrating synergism *in vitro* with these same drug combinations.

*In vitro* experiments allow a qualitative and quantitative analysis of the results based on the mathematical methods developed by Chou and Talalay (ibid).

The mathematical method by Chou and Talalay is based on the Median-Effect-Principle that can accurately describe the experimental findings in a qualitative and quantitative manner. For mutually exclusive drugs, they showed that the generalized isobol equation applies for any degree of effect (see page 52 in Chou and Talalay). An isobol or isobologram is the graphic representation of all dose combinations of two drugs that have the same degree of effect, for example all combinations of x amounts of drug A and y amounts of drug B that will achieve the same degree of cell kill, such as 20% or 50% cell kill. The equation is valid for any degree of effect and the graphic representation will have the same shape (page 54, line 1, in Chou and Talalay), which is presented in Figure 11 D (page 51 in Chou and Talalay).

In isobolograms, a straight line indicates additive effects, a concave curve (curve below the straight line) represents synergistic effects, and a convex curve (curve above the straight line) represents antagonistic effects. **These curves show that a combination of two mutually exclusive drugs will yield the same type of effect over the whole concentration range and at any ratio of the two drugs** (the concave and convex curves will not cross the diagonal line).

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Thus, the use of two cytotoxic drugs with different killing mechanisms demonstrates either an additive, synergistic, or antagonistic effect.

For the quantitation of the observed synergistic effect in an *in vitro* system, Chou and Talalay (Chapter 5, page 35 ff) developed the combination index (CI) method. In this method, a CI of 1 indicates an additive effect, a CI of  $< 1$  indicates synergism, and a CI of  $> 1$  indicates antagonism. This method allows a rapid experimental approach for the evaluation of the combined effect of two mutually exclusive drugs. We applied the CI method for the performance of the *in vitro* studies and the analysis of the results.

The following experiments were performed under my direct supervision.

#### **Experimental Methods:**

To determine the cytotoxicity of huN901-DM1 in combination with dexamethasone, melphalan, oxaliplatin, etoposide and camptothecin, multiple myeloma cell lines MOLP8 and NCI H929 were used. The SW2 small cell lung carcinoma cell line was applied for combination studies of huN901-DM1 with taxol.

Cells in exponential growth were seeded in 96-well plates at a density of either 2,000 cells per well (SW2) or 2,500 cells (MOLP8 and NCI H929). Immediately after seeding, the cells were exposed to various concentrations of the conjugate, a small drug, or their combination. After either a four-day (SW2) or a six-day (MOLP8 and NCI H929, combination studies with melphalan) incubation period, a WST-8 cell proliferation assay (Dojindo Laboratories, Gaithersburg, MD) was performed. For combination experiments of huN901-DM1 with oxaliplatin, etoposide and camptothecin on multiple myeloma cell lines (MOLP8 and NCI

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H929), the incubation period was four days, after which an Alamar blue proliferation assay was performed (Biosource, Camarillo, CA).

Results were expressed as survival fractions (the ratio of treated vs. untreated cells). Experiments were run two or three times, and each experiment was performed in triplicate. Data were analyzed using the median effect analysis (Chou and Talalay, 1984), using the commercially available software CompuSyn (ComboSyn Inc., Paramus, NJ). Combination indices (CI) for independent experiments were calculated separately and results of all experiments are reported. Abbreviations: CI: combination index, Fa: fraction affected (fraction of killed cells).

**Results:**

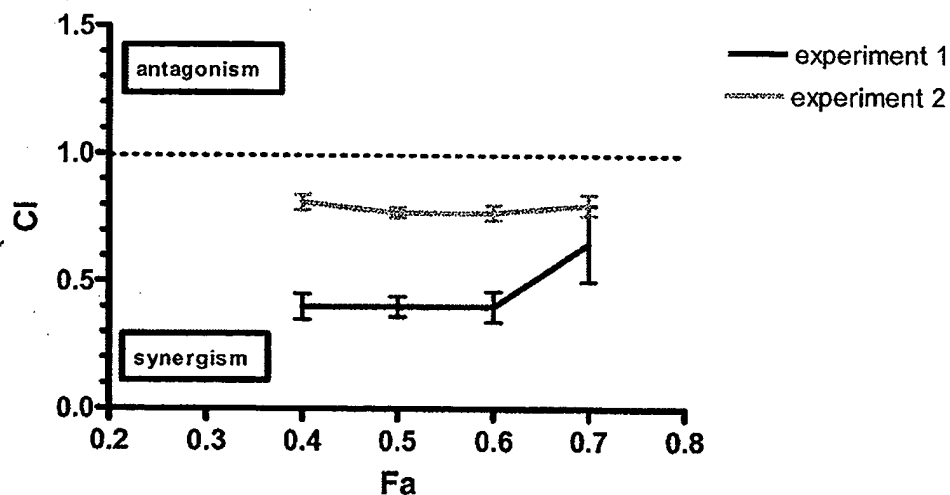
**Table:** Summary of results of *in vitro* treatments of three different cell lines with the immunoconjugate, huN901-DM1, in combination with different cytotoxic agents. The results show synergistic effects for the combinations.

Drug combination		Drug combination effect on three cell lines		
		NCI H929	MOLP8	SW2
1	huN901-DM1 + camptothecin	moderately synergistic	synergistic	
2	huN901-DM1 + oxaliplatin	synergistic	synergistic	
3	huN901-DM1 + etoposide	synergistic	synergistic	
4	huN901-DM1 + taxol			synergistic

**Experimental results of drug combination studies.** The combination index (CI) as a function of the Fraction affected (Fa) was determined as described under the *Experimental Methods* section. The results are presented in tables and as plots of CI vs. Fa.

**Drug Combination 1: huN901-DM1 + camptothecin on MOLP8 cells**

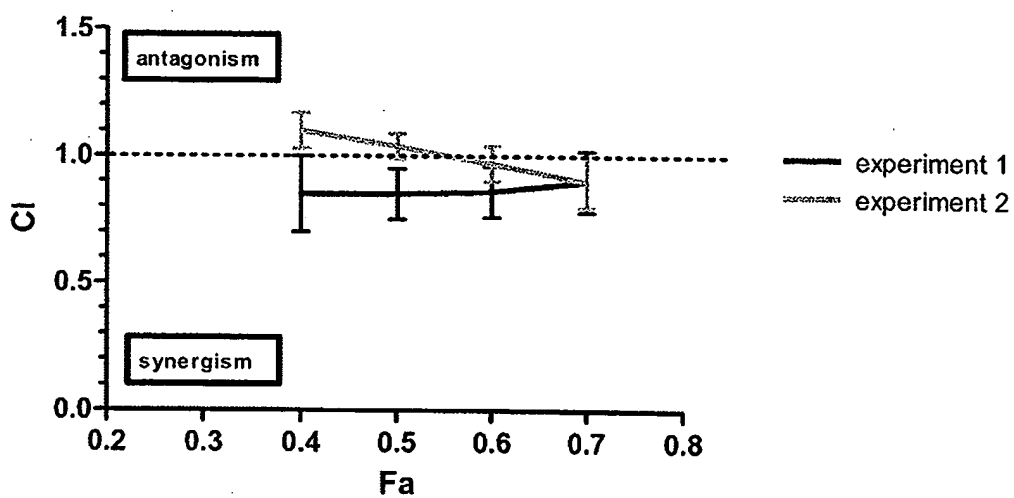
Fraction affected (Fa)	CI (SD estimated by the sequential deletion analysis)	
	Experiment 1	Experiment 2
0.3	0.53 (0.10)	0.90 (0.04)
0.4	0.36 (0.04)	0.80 (0.03)
0.5	0.30 (0.04)	0.75 (0.02)
0.6	0.36 (0.05)	0.75 (0.03)
0.7	0.65 (0.15)	0.76 (0.04)



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**Drug combination 1: huN901-DM1 + camptothecin on NCI H929 cells**

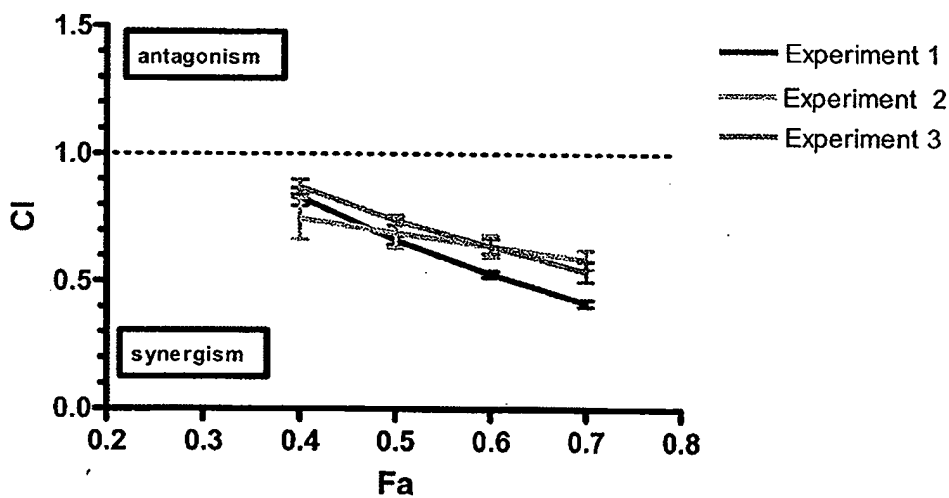
Fraction affected	CI, (SD estimated by the sequential deletion analysis)	
	Experiment 1	Experiment 2
0.4	0.85 (0.15)	1.10 (0.07)
0.5	0.85 (0.10)	1.04 (0.05)
0.6	0.86 (0.10)	0.97 (0.07)
0.7	0.90 (0.12)	0.90 (0.10)



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**Drug Combination 2: huN901-DM1 + oxaliplatin on NCI H929 cells**

Fraction affected	CI, (SD estimated by the sequential deletion analysis)		
	Experiment 1	Experiment 2	Experiment 3
0.3	1.07 (0.07)	0.81 (0.11)	1.03 (0.05)
0.4	0.80 (0.03)	0.74 (0.08)	0.87 (0.02)
0.5	0.66 (0.02)	0.68 (0.06)	0.74 (0.02)
0.6	0.53 (0.02)	0.63 (0.04)	0.64 (0.02)
0.7	0.41 (0.02)	0.58 (0.04)	0.54 (0.04)

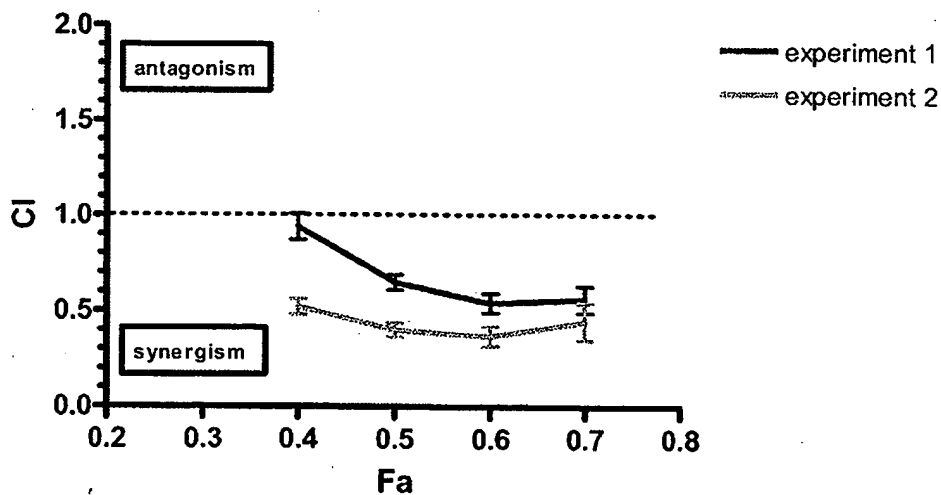




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**Drug Combination 2: huN901-DM1 + oxaliplatin on MOLP8 cells**

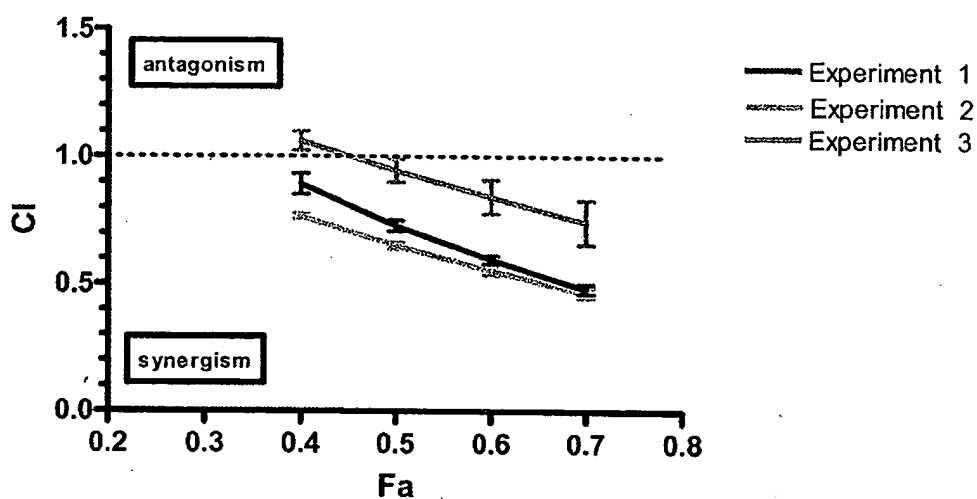
Fraction affected	CI, (SD estimated by the sequential deletion analysis)	
	Experiment 1	Experiment 2
0.3	1.60 (0.20)	0.77 (0.10)
0.4	0.94 (0.07)	0.52 (0.04)
0.5	0.65 (0.05)	0.40 (0.03)
0.6	0.55 (0.05)	0.37 (0.05)
0.7	0.56 (0.07)	0.44 (0.10)



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**Drug Combination 3: huN901-DM1 + etoposide on NCI H929 cells**

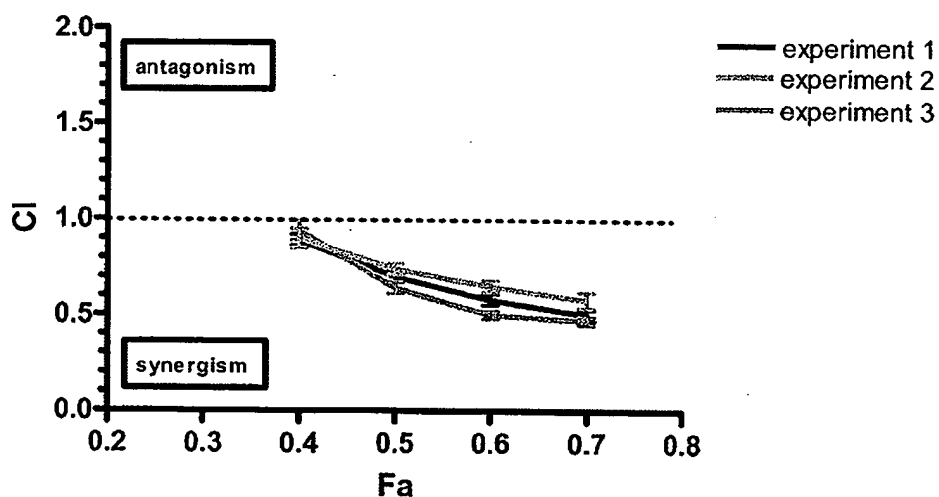
Fraction affected	CI, (SD estimated by the sequential deletion analysis)		
	Experiment 1	Experiment 2	Experiment 3
0.3	1.10 (0.08)	0.91 (0.01)	1.2 (0.08)
0.4	0.90 (0.04)	0.76 (0.01)	1.00 (0.04)
0.5	0.73 (0.02)	0.65 (0.01)	0.90 (0.04)
0.6	0.60 (0.01)	0.55 (0.01)	0.84 (0.07)
0.7	0.48 (0.02)	0.47 (0.02)	0.74 (0.09)



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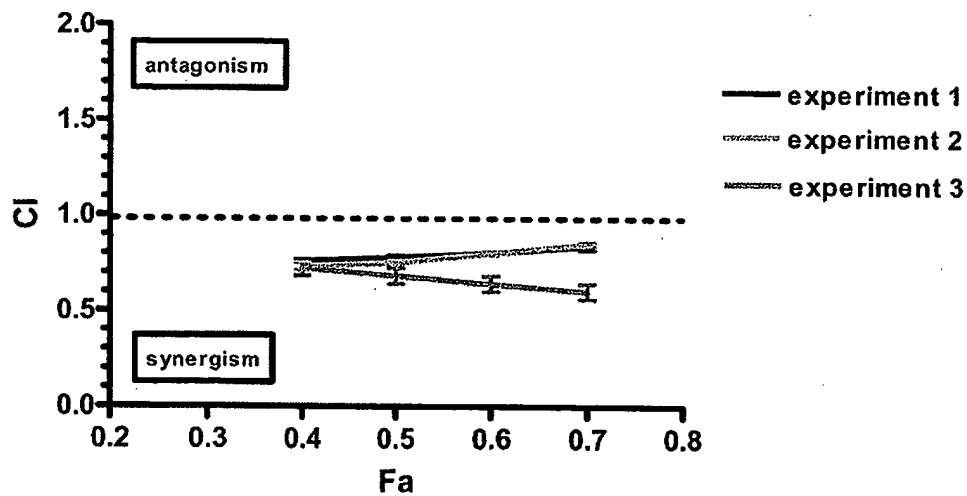
**Drug Combination 3: huN901-DM1 + etoposide on MOLP 8 cells**

Fraction affected	CI, (SD estimated by the sequential deletion analysis)		
	Experiment 1	Experiment 2	Experiment 3
0.3	1.20 (0.07)	1.08 (0.10)	1.4 (0.12)
0.4	0.90 (0.04)	0.88 (0.05)	0.94 (0.05)
0.5	0.70 (0.03)	0.75 (0.03)	0.64 (0.03)
0.6	0.58 (0.03)	0.65 (0.03)	0.50 (0.02)
0.7	0.50 (0.03)	0.58 (0.04)	0.47 (0.02)



**Drug Combination 4: huN901-DM1+taxol on SW2 cells**

Fraction affected	CI, (SD estimated by the sequential deletion analysis)		
	Experiment 1	Experiment 2	Experiment 3
0.3	0.732 (0.02)	0.75 (0.10)	0.75 (0.06)
0.4	0.75 (0.01)	0.77 (0.01)	0.72 (0.04)
0.5	0.78 (0.01)	0.79 (0.01)	0.68 (0.04)
0.6	0.80 (0.01)	0.80 (0.01)	0.64 (0.04)
0.7	0.84 (0.02)	0.83 (0.01)	0.60 (0.04)




I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States

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Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: May 15, 2006

  
Walter A. Blättler, Ph.D.

HU and Y.-C. CHENG

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## QUANTITATIVE ANALYSIS OF DOSE-EFFECT RELATIONSHIPS: THE COMBINED EFFECTS OF MULTIPLE DRUGS OR ENZYME INHIBITORS

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### INTRODUCTION

The quantitative relationship between the dose or concentration of a given ligand and its effect is a characteristic and important descriptor of many biological systems varying in complexity from isolated enzymes (or binding proteins) to intact animals. This relationship has been analyzed in considerable detail for reversible inhibitors of enzymes. Such analyses have made assumptions on the mechanism of inhibition (competitive, noncompetitive, uncompetitive), and on the mechanism of the reaction for multi-substrate enzymes (sequential or ping-pong), and have required knowledge of kinetic constants (1-4). More recently, it has been possible to describe the behavior of such enzyme inhibitors by simple generalized equations that are independent of inhibitor or reaction mechanisms and do not require knowledge of conventional kinetic constants (i.e.  $K_m$ ,  $K_i$ ,  $V_{max}$ ) (5-8).

Our understanding of dose-effect relationships in pharmacological systems has not advanced to the same level as those of enzyme systems. Many types of mathematical transformations have been proposed to linearize dose-effect plots, based on statistical or empirical assumptions, e.g. probit (9, 10), logit (11) or power-law functions (12). Although these methods often provide adequate linearizations of plots, the slopes and intercepts of such graphs are usually devoid of any fundamental meaning.

### THE MEDIAN EFFECT PRINCIPLE

We demonstrate here the application of a single and generalized method for analyzing dose-effect relationships in enzymatic, cellular and whole animal systems. We also examine the problem of quantitating the effects of multiple inhibitors on such systems and provide definitions of summation of effects, and consequently of synergism and antagonism.

Since the proposed method of analysis is derived from generalized mass action considerations, we caution the reader that the analysis of dose-effect

data is concerned with basic mass-action characteristics rather than with proof of specific mechanisms. Nevertheless, it is convenient and intuitively attractive to analyze and normalize all types of dose-response results by a uniform method which is based on sound fundamental considerations that have physicochemical and biochemical validity in simpler systems. Our analysis is based on the median effect principle of the mass action law (5-8), and has already been shown to be simple to apply and useful in the analysis of complex biological systems (13).

#### *The Median Effect Equation*

The median effect equation (6, 8) states that:

$$f_a/f_u = (D/D_m)^m \quad (1)$$

where  $D$  is the dose,  $f_a$  and  $f_u$  are the fractions of the system affected and unaffected, respectively, by the dose  $D$ ,  $D_m$  is the dose required to produce the median effect (analogous to the more familiar  $IC_{50}$ ,  $ED_{50}$ , or  $LD_{50}$  values), and  $m$  is a Hill-type coefficient signifying the sigmoidicity of the dose-effect curve, i.e.,  $m = 1$  for hyperbolic (first order or Michaelis-Menten) systems. Since by definition,  $f_a + f_u = 1$ , several useful alternative forms of equation 1 are:

$$f_a/(1 - f_a) = [(f_a)^{-1} - 1]^{-1} = [(f_u)^{-1} - 1] = (D/D_m)^m$$

$$f_a = 1/[1 + (D_m/D)^m]$$

$$D = D_m[f_a/(1 - f_a)]^{1/m}$$

The median effect equation describes the behavior of many biological systems. It is, in fact, a generalized form of the enzyme kinetic relations of Michaelis-Menten (14) and Hill (15), the physical adsorption isotherm of Langmuir (16), the pH-ionization equation of Henderson and Hasselbalch (17), the equilibrium binding equation of Scatchard (18), and the pharmacological drug-receptor interaction (19). Furthermore, the median effect equation is directly applicable not only to primary ligands such as substrates, agonists, and activators, but also to secondary ligands such as inhibitors, antagonists, or environmental factors (5, 6).

When applied to the analysis of the inhibition of enzyme systems, the median effect equation can be used without knowledge of conventional kinetic constants (i.e.  $K_m$ ,  $V_{max}$  or  $K_i$ ) and irrespective of the mechanism of inhibition (i.e. competitive, noncompetitive or uncompetitive). Furthermore, it is valid for multisubstrate reactions irrespective of mechanism (sequential or ping-pong) (5-8).

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### The Median Effect Plot

The median effect equation (equation 1) may be linearized by taking the logarithms of both sides, i.e.

$$\begin{aligned} \log (f_i/f_0) &= m \log (D) - m \log (D_m) \\ \text{or} \quad \log [(f_i)^{-1} - 1]^{-1} &= m \log (D) - m \log (D_m) \\ \text{or} \quad \log [(f_i)^{-1} - 1] &= m \log (D) - m \log (D_m) \end{aligned}$$

The median effect plot (Fig. 1) of  $y = \log (f_i/f_0)$  or its equivalents with respect to  $x = \log (D)$  is a general and simple method (13, 30) for determining pharmacological median doses for lethality ( $LD_{50}$ ), toxicity ( $TD_{50}$ ), effect of agonist drugs ( $ED_{50}$ ), and effect of antagonist drugs ( $IC_{50}$ ). Thus, the median-effect principle of the mass-action law encompasses a wide range of applications. The plot gives the slope,  $m$ , and the intercept of the dose-effect plot with the median-effect axis [i.e. when  $f_i = f_0$ ,  $f_i/f_0 = 1$  and hence  $y = \log (f_i/f_0) = 0$ ] which gives  $\log (D_m)$  and consequently the  $D_m$  value. Any cause-consequence relationship that gives a straight line for this plot will provide the two basic parameters,  $m$  and  $D_m$ , and thus, an apparent equation that describes such a system. The linearity of the median-effect plot (as determined from linear regression coefficients) determines the applicability of the present method.

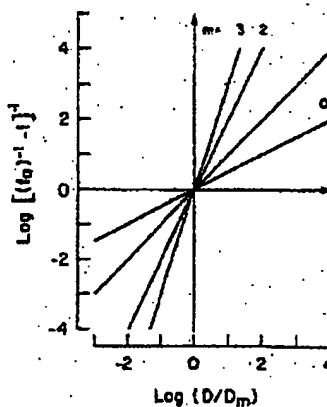


FIG. 1. The median-effect plot at different slopes corresponding to  $m$  values of 0.5, 1, 2 and 3. The plot is based on the median-effect equation (equation 1) in which the dose ( $D$ ) has been normalized by taking the ratio to the median-effect dose ( $D_m$ ). Note that the ordinate  $\log [(f_i)^{-1} - 1]^{-1}$  is identical to  $\log [(f_i)^{-1} - 1]$  or  $\log (f_i/f_0)$ .



### *Relation of the Median-Effect Equation to Michaelis-Menten and Hill Equations*

In the special case, when  $m = 1$ , equation 1 becomes  $f_x = [1 + (D_m/D)]^{-1}$  which has the same form as the Michaelis-Menten equation (14),  $v/V_{max} = [1 + (K_m/S)]^{-1}$ . In addition, when the effector ligand is an environmental factor such as an inhibitor, the equation,  $f_x = [1 + (D_m/D)]^{-1}$ , is valid not only for a single substrate reaction (Michaelis-Menten equation) but also for multiple substrate reactions; the fractional effect is expressed with respect to the control velocity rather than to the maximal velocity (6). Furthermore,  $f_x$  in equation 1 is simple to obtain, whereas the determination of  $V_{max}$  in the Michaelis-Menten (or Hill) equations requires approximation or extrapolation (6, 7). The logarithmic form of equation 1 describes the Hill equation.

### *The Utility of the Median Effect Principle*

The median-effect equation has been used to obtain accurate values of  $IC_{50}$ ,  $ED_{50}$ ,  $LD_{50}$ , or the relative potencies of drugs or inhibitors in enzyme systems (6-8, 21-26), in cellular systems (20, 27, 28) and in animal systems (13, 29-32). An alternative form of the median-effect equation (5) has been used for calculating the dissociation constant ( $K_i$  or  $K_a$ ) of ligands for pharmacological receptors (33-35). It has also permitted the analysis of chemical carcinogenesis data and has predicted especially accurately tumor incidence at low dose carcinogen exposure (30, 31). By using the median-effect principle, the general equation for describing a standard radioimmunoassay or ligand displacement curve has been derived recently by Smith (36). It has also been used to show that there is marked synergism among chemotherapeutic agents in the treatment of hormone-responsive experimental mammary carcinomas (32). In recent preliminary reports (13, 37), we have shown that, in conjunction with the multiple drug effect equations (see below), the median-effect plot forms the basis for the quantitation of synergism, summation and antagonism of drug effects.

## ANALYSIS OF MULTIPLE DRUG EFFECTS

### *An Overview*

Over the past decades, numerous authors have claimed synergism, summation or antagonism of the effects of multiple drugs. However, there is still no consensus as to the meanings of these terms. For instance, in a review, Goldin and Mantel in 1957 (38) listed seven different definitions of these terms. Confusion and ambiguity persist (39) despite increasing use of multiple drugs in experimentation and in therapy. This emphasizes the lack of a

PAUL TALALAY

*Michaelis-Menten and Hill*

Equation (14) becomes  $f_i = [1 + (D_m/D)^i]^{-1}$ . Michaelis-Menten (14),  $v/V_{max} = [1 + (D_m/D)]^{-1}$ , is an environmental factor. Hill (15) is valid not only for a 1:1 system (16) but also for multiple systems (17) with respect to the Hill equation (16). Furthermore,  $f_i$  in the Hill equation (16) is an approximation or extrapolation of the Hill equation.

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## EFFECTS

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theoretical basis that would permit rigorous and quantitative assessment of the effects of drug combinations.

Attempts to interpret the effect of multiple drugs have been documented for more than a century (39). Since the introduction of the isobol concept by Loewe in 1928 (40, 41) and the fractional product concept (see Appendix) by Webb in 1963 (42), the theoretical and practical aspects of the problem have been the subject of many reviews (38, 43-51). Some authors have discussed the possible mechanisms that may lead to synergism, and others have emphasized methods of data analysis. The kinetic approach was used earlier by some investigators (4, 42, 52-58), but the formulations were frequently too complex to be of practical usefulness or were restricted to individual situations. Although not specifically stated, some formulations are limited to two inhibitors; others are valid only for first order (Michaelis-Menten type) systems but not for higher order (Hill type) systems, and still others are valid only for mutually nonexclusive inhibitors but not for mutually exclusive inhibitors.

The present authors, therefore, have undertaken a kinetic approach to analyze the problem. An unambiguous definition of summation is a prerequisite for any meaningful conclusions with respect to synergism and antagonism. Ironically, two prevalent concepts for calculating summation i.e., the isobol and the fractional-product method, are shown to conform to two opposite situations. The former concept is valid for drugs whose effects are mutually exclusive, and the latter is valid for mutually nonexclusive drugs (13, 49), and thus these methods cannot be used indiscriminately (see Appendix). In this paper, we provide the equations for both situations and show that they are merely special cases of the general equations described recently (59). We also propose a general diagnostic plot to determine the applicability of experimental data, to distinguish mutually exclusive from nonexclusive drugs, and to obtain parameters that can be directly used for the analysis of summation, synergism or antagonism.

*Requirements for Analyzing Multiple Drug Effects*

The following information is essential for analyzing multiple drug effects and for quantitating synergism, summation and antagonism of multiple drugs.

1. A quantitative definition of summation is required since synergism implies more than summation and antagonism less than summation of effects.

2. Dose-effect relationships for drug 1, drug 2 and their mixture (at a known ratio of drug 1 to drug 2) are required.

a. Measurements made with single doses of drug 1, drug 2 and their mixture can never alone determine synergism since the sigmoidicity of dose-effect

curves and the exclusivity of drug effects cannot be determined from such measurements.

b. The dose-effect relationships should follow the basic mass-action principle relatively well (e.g. median-effect plots with correlation coefficients for the regression lines greater than 0.9).

c. Determination of the sigmoidicity of dose-effect curves and the exclusivity of effects of multiple drugs is necessary. The slope of the median-effect plot gives a quantitative estimation of sigmoidicity. When  $m = 1$ , the dose-effect curve is hyperbolic; when  $m \neq 1$ , the dose-effect curve is sigmoidal, and the greater the  $m$  value, the greater its sigmoidicity;  $m < 1$  is a relatively rare case which in allosteric systems indicates negative cooperativity of drug binding at the receptor sites. When the dose-effect relationships of drug 1, drug 2 and their mixture are all parallel in the median-effect plot, the effects of drug 1 and drug 2 are mutually exclusive (59). If the plots of drugs 1 and 2 are parallel but the plot of their mixture is concave upward with a tendency to intersect the plot of the more potent of the two drugs, their effects are mutually nonexclusive (59). If the plots for drugs 1 and 2 and their mixture are not parallel to each other, exclusivity of effects cannot be established. Alternatively, exclusivity of effects may not be ascertained because of a limited number of data points or limited dose range. In these cases, the data may be analyzed for the "combination index" (see below) on the basis of both mutually exclusive and mutually nonexclusive assumptions. Note that exclusivity may occur at a receptor site, at a point in a metabolic pathway, or in more complex systems, depending on the endpoint of the measurements.

#### *Equations for the Effects of Multiple Drugs*

A systematic analysis in enzyme kinetic systems using the basic principles of the mass action law has led to the derivation of generalized equations for multiple inhibitors or drugs (8, 59).

1. *For two mutually exclusive drugs that obey first order conditions.* If two drugs (e.g., inhibitors  $D_1$  and  $D_2$ ) have effects that are mutually exclusive, then the summation of combined effects  $(\xi)_{1,2}$  in first-order systems (i.e., each drug follows a hyperbolic dose-effect curve) can be calculated from (59):

$$\begin{aligned} \frac{(\xi)_{1,2}}{(\xi)_h} &= \frac{(\xi)_1}{(\xi)_h} + \frac{(\xi)_2}{(\xi)_h} \\ &= \frac{(D)_1}{(ED)_{50,1}} + \frac{(D)_2}{(ED)_{50,2}} \end{aligned} \quad (2)$$

cannot be determined from such

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effect plots with correlation coefficients

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necessary. The slope of the median-  
on of sigmoidicity. When  $m = 1$ , the  
 $m \neq 1$ , the dose-effect curve is  
the greater its sigmoidicity;  $m < 1$  is a  
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in first-order systems (i.e., each drug  
can be calculated from (59):

$$\frac{1}{f_{0.5}} + \frac{(f_2)_2}{(f_0)_2} = \frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} \quad (2)$$

where  $f_a$  is the fraction affected and  $f_u$  is the fraction unaffected, and  $ED_{50}$  is the concentration of the drug that is required to produce a 50% effect. Note that  $f_a + f_u = 1$  or  $f_u = 1 - f_a$ .

2. For two mutually nonexclusive drugs that obey first order conditions. If the effects of two drugs ( $D_1$  and  $D_2$ ) are mutually non-exclusive (i.e., they have different modes of action or act independently) the summation of combined effects,  $(f_a)_{1,2}$ , in a first-order system is (59):

$$\begin{aligned} \frac{(f_a)_{1,2}}{(f_u)_{1,2}} &= \frac{(f_a)_1}{(f_u)_1} + \frac{(f_a)_2}{(f_u)_2} + \frac{(f_a)_1 (f_a)_2}{(f_u)_1 (f_u)_2} \\ &= \frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} + \frac{(D)_1 (D)_2}{(ED_{50})_1 (ED_{50})_2} \quad (3) \end{aligned}$$

Similar relationships apply to situations involving more than two inhibitors, for which generalized equations are given in ref. 59. In enzyme systems, equations 2 and 3 express summation of inhibitory effects, irrespective of the number of substrates, the type or mode of reversible inhibition (competitive, noncompetitive or uncompetitive) or the kinetic mechanisms (sequential or ping-pong) of the reaction under consideration. The simplicity of the above equations (in which all specific kinetic constants, substrate concentration factors, and  $V_{max}$  have been cancelled out during derivation) suggests their general applicability (5, 6). This is in contrast to the mechanism-specific reactions (3, 5) for which the equations are far more complex. In more organized cellular or animal systems, the dose-effect relationships of drugs or inhibitors are frequently sigmoidal rather than hyperbolic.

3. For two mutually exclusive drugs that obey higher order conditions. The above concepts have been extended to higher-order (Hill-type) systems in which each drug has a sigmoidal dose-effect curve (i.e., has more than one binding site or exhibits positive or negative cooperativity). If the effects of such drugs are mutually exclusive:

$$\left[ \frac{(f_a)_{1,2}}{(f_u)_{1,2}} \right]^{\frac{1}{m}} = \left[ \frac{(f_a)_1}{(f_u)_1} \right]^{\frac{1}{m}} + \left[ \frac{(f_a)_2}{(f_u)_2} \right]^{\frac{1}{m}} = \frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} \quad (4)$$

where  $m$  is a Hill-type coefficient which denotes the sigmoidicity of the dose-effect curve.

4. For two mutually nonexclusive drugs that obey higher order conditions. If the effects of two drugs ( $D_1$  and  $D_2$ ) are mutually nonexclusive and if each drug and their combination follow a sigmoidal dose-effect relationship with  $m^{\text{th}}$  order kinetics, then this relationship becomes (59):

$$\begin{aligned} \left[ \frac{(f_1)_{1,2}}{(f_1)_{1,2}} \right]^{\frac{1}{m}} &= \left[ \frac{(f_1)_1}{(f_1)_1} \right]^{\frac{1}{m}} + \left[ \frac{(f_1)_2}{(f_1)_2} \right]^{\frac{1}{m}} + \left[ \frac{(f_1)_1 (f_1)_2}{(f_1)_1 (f_1)_2} \right]^{\frac{1}{m}} \\ &= \frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} + \frac{(D)_1 (D)_2}{(ED_{50})_1 (ED_{50})_2} \quad (5) \end{aligned}$$

In the special case where  $(f_1)_{1,2} = 0.5$ , equations 2 and 4 become:

$$\frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} = 1 \quad (6)$$

which describes the  $ED_{50}$  isobologram.

Similarly, equations 3 and 5 become:

$$\frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} + \frac{(D)_1 (D)_2}{(ED_{50})_1 (ED_{50})_2} = 1 \quad (7)$$

which does not describe an isobologram, because of the additional term on the left.

In the Appendix it is shown that equation 3 or 7 can be readily used for deriving the fractional product equation of Webb (42), and equation 4 can be used for deriving the generalized isobologram equation for any desired  $f$  value. Thus, for the isobologram of any fractional effect  $f = x$  per cent, the generalized equation is:

$$\frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} = 1 \quad (8)$$

The limitations of the fractional product concept and the isobologram method are detailed in the Appendix.

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that obey higher order conditions. If mutually nonexclusive and if each individual dose-effect relationship with becomes (59):

$$\left[ \frac{f_h}{h} \right]^{\frac{1}{m}} + \left[ \frac{(f_h)_1 (f_h)_2}{(L_h)_1 (L_h)_2} \right]^{\frac{1}{m}}$$

$$\frac{(D_h)_1 (D_h)_2}{(ED_{50})_1 (ED_{50})_2} + \frac{(D_h)_1 (D_h)_2}{(ED_{50})_1 (ED_{50})_2} \quad (5)$$

if  $\alpha \approx 0.5$ , equations 2 and 4 become:

$$\frac{(D_h)_1 (D_h)_2}{(ED_{50})_1 (ED_{50})_2} = 1 \quad (6)$$

$$\frac{(D_h)_1 (D_h)_2}{(ED_{50})_1 (ED_{50})_2} = 1 \quad (7)$$

because of the additional term on the equation 3 or 7 can be readily used for of Webb (42), and equation 4 can be isobologram equation for any desired  $f_x$  fractional effect  $f_x = x$  per cent, the

$$\frac{(D_h)_1 (D_h)_2}{(D_h)_1 (D_h)_2} = 1 \quad (8)$$

adduct concept and the isobologram

5. Quantitation of synergism, summation and antagonism. When experimental results are entered into equations 2-5, if the sum of the two terms in equation 2 or 4, or the sum of the three terms in equation 3 or 5 is greater than, equal to, or smaller than 1, it may be inferred that antagonism, summation or synergism of effects, respectively, has been observed. Therefore, from equations 2-5, if the combined observed effect is greater than the calculated additive effect,  $(f_x)_{1,2}$ , synergism is indicated; if it is smaller, antagonism is indicated.

It is, however, convenient to designate a "combination index" (CI) for quantifying synergism, summation, and antagonism, as follows:

$$CI = \frac{(D_h)_1}{(D_h)_1} + \frac{(D_h)_2}{(D_h)_2} \quad (9)$$

for mutually exclusive drugs, and

$$CI = \frac{(D_h)_1}{(D_h)_1} + \frac{(D_h)_2}{(D_h)_2} + \frac{(D_h)_1 (D_h)_2}{(D_h)_1 (D_h)_2} \quad (10)$$

for mutually nonexclusive drugs.

For mutually exclusive or nonexclusive drugs,

when  $CI < 1$ , synergism is indicated.

$CI = 1$ , summation is indicated.

$CI > 1$ , antagonism is indicated.

To determine synergism, summation and antagonism at any effect level (i.e., for any  $f_x$  value), the procedure involves three steps: i) Construct the median-effect plot (Eqn. 1) which determines  $m$  and  $D_m$  values for drug 1, drug 2 and their combination; ii) for a given degree of effect (i.e., a given  $f_x$  value representing  $x$  per cent affected), calculate the corresponding doses [i.e.,  $(D_h)_1$ ,  $(D_h)_2$  and  $(D_h)_{1,2}$ ] by using the alternative form of equation 1,  $D_x = D_m [f_x / (1 - f_x)]^{1/m}$ ; iii) calculate the combination index (CI) by using equations 9 or 10; where  $(D_h)_1$  and  $(D_h)_2$  are from step (ii), and  $(D_h)_{1,2}$  [also from step (ii)] can be dissected into  $(D_h)_1$  and  $(D_h)_2$  by their known ratio,  $P/Q$ . Thus,  $(D_h)_1 = (D_h)_{1,2} \times P / (P + Q)$  and  $(D_h)_2 = (D_h)_{1,2} \times Q / (P + Q)$ . CI values that are smaller than, equal to, or greater than 1, represent synergism, summation and antagonism, respectively.

To facilitate the calculation, a computer program written in BASIC for automatic graphing of CI with respect to  $f_x$  has been developed. Samples of this computer simulation are shown in the examples to be given later. A sample calculation of CI without using a computer is also given in Example 1.

# APPLICATIONS OF THE MEDIAN EFFECT EQUATION AND PLOTS TO THE ANALYSIS OF MULTIPLE DRUGS OR INHIBITORS

## Example 1. Inhibition of Alcohol Dehydrogenase by Two Mutually Exclusive Inhibitors

Yonetani and Theorell (55) have reported the inhibition of horse liver alcohol dehydrogenase by two inhibitors ( $I_1$  = ADP-ribose and  $I_2$  = ADP) both of which are competitive with respect to NAD. Velocity measurements in the presence of a range of concentrations of the two inhibitors (alone and in combination) and control velocities were retrieved from the original plot, and tabulated in ref. 59. The results are most conveniently expressed as fractional velocities ( $f$ ), which are the ratios of the inhibited velocities to the control velocities, and therefore correspond to the fraction of the process unaffected ( $f_0$ ). The fractional velocities in the presence of ADP-ribose (95–375  $\mu$ M), ADP (0.5–2.5  $\mu$ M), and a combination of ADP-ribose and ADP at a constant molar ratio of 190:1, have been plotted as  $\log[(f_0/f)^{-1} - 1]$  with respect to  $\log(I)$  (Fig. 2). For ADP-ribose,  $m = 0.968$ ,  $I_{50} = 156.1 \mu$ M with a regression coefficient of  $r = 0.9988$ . For ADP,  $m = 1.043$ ,  $I_{50} = 1.657 \mu$ M and  $r = 0.9996$ . For ADP-ribose and ADP in combination (molar ratio 190:1),  $m_{1,2} = 1.004$ ,  $(I_{50})_{1,2} = 107.0 \mu$ M and  $r = 0.9997$ . It is clear that both inhibitors follow first-order kinetics (i.e.,  $m \approx 1$ ) and that ADP-ribose and ADP are mutually exclusive inhibitors (i.e., the

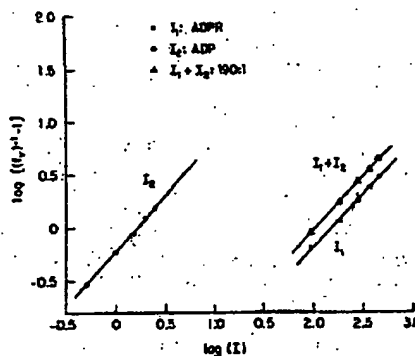
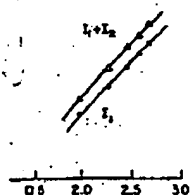


FIG. 2. Median-effect plots of the experimental data of Yonetani and Theorell (55) for the inhibition of horse liver alcohol dehydrogenase by two mutually exclusive inhibitors.  $I_1$  is ADP-ribose (ADPR),  $I_2$  is ADP, and  $I_1 + I_2$  is a mixture of ADP-ribose and ADP in a molar ratio of 190:1. The abscissa represents  $\log(I_1)$  ( $\bullet$ ),  $\log(I_2)$  ( $\circ$ ), or  $\log[(I_1 + I_2)/190:1]$  ( $\Delta$ ). In this case it is convenient to use the terms fractional velocity ( $f$ ) which is the ratio of the inhibited to the control velocity and therefore corresponds to the fraction that is unaffected ( $f_0$ ). [from Chou and Talalay (59)].

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## genase by Two Mutually Exclusive

orted the inhibition of horse liver  
s ( $I_1$  = ADP-ribose and  $I_2$  = ADP)  
t to NAD. Velocity measurements in  
of the two inhibitors (alone and in  
retrieved from the original plot, and  
conveniently expressed as fractional  
e inhibited velocities to the control  
re fraction of the process unaffected  
ce of ADP-ribose (95–375  $\mu$ M), ADP  
-ribose and ADP at a constant molar  
): 1 – 1] with respect to  $\log(I)$  (Fig. 2).  
m with a regression coefficient of  $r =$   
 $\mu$ M and  $r = 0.9996$ . For ADP-ribose  
0:1),  $m_{1,2} = 1.004$ ,  $(I_{50})_{1,2} = 107.0 \mu$ M  
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data of Yonetani and Theorell (55) for the  
two mutually exclusive inhibitors.  $I_1$  is ADP-  
of ADP-ribose and ADP in a molar ratio of  
) $\lambda$  or  $\log[(I_1) + (I_2)\lambda(190:1; \Delta)]$ . In this case it is  
which is the ratio of the inhibited to the control  
that is unaffected ( $I_1$ ). (from Chou and Talalay

plot for the combination of inhibitors parallels the plots for each of the  
component inhibitors). These conclusions are in agreement with the  
interpretations obtained by Yonetani and Theorell (55) and Chou and Talalay  
(59) using different methods. For the present analysis, knowledge of kinetic  
constants and type of inhibition is not required. The plots show excellent  
agreement between theory and experiment.

With this knowledge of the  $m$  and  $I_{50}$  values for each inhibitor and the  
combination at a constant molar ratio, it is possible to calculate the inhibitor  
combination index (CI) for a series of values of  $f_1$  (Fig. 3). The CI values are  
close to 1 over the entire range of  $f_1$  values, suggesting strongly that the  
inhibitory effects of ADP-ribose and ADP are additive.

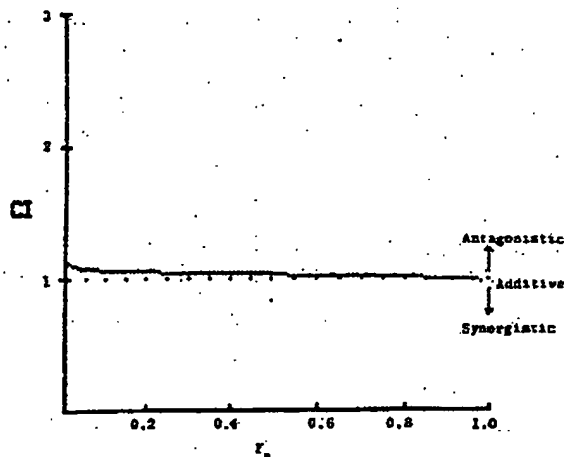


FIG. 3. Computer-generated graphical presentation of the combination index (CI) with respect to  
fraction affected ( $f_1$ ) for the additive inhibition by ADP-ribose and ADP (molar ratio of 190:1) of  
horse liver alcohol dehydrogenase. The plot is based on equation 9 (mutually exclusive) as  
described in the section entitled "Quantitation of Synergism, Summation and Antagonism." CI is  
the combination index which is equal to  $(D_1)/(D_{1,2}) + (D_2)/(D_{2,1})$  (see text for sample calculation).  
 $CI < 1$ ,  $= 1$  and  $> 1$  represent synergistic, additive and antagonistic effects, respectively.  
Although plots of CI with respect to  $f_1$  can be obtained by step-by-step calculations, it is much  
more convenient to use computer simulation. The parameters were obtained as described in Fig.  
2, by the use of linear regression analysis or computer simulation.

We now give a sample calculation of the combination index (CI) for an  
arbitrarily selected value of  $f_1 = 0.9$ :

From equation 1,  $D_1 = D_m [f_1/(1 - f_1)]^{1/m}$ .

Since  $I_1$  is ADP-ribose and  $I_2$  is ADP,

then  $(D_{50})_1 = 156.1 \mu\text{M} [0.9/(1 - 0.9)]^{1/1.004} = 1511 \mu\text{M}$

$(D_{50})_2 = 1.657 \mu\text{M} [0.9/(1 - 0.9)]^{1/1.004} = 13.62 \mu\text{M}$

$(D_{50})_{1,2} = 107.0 \mu\text{M} [0.9/(1 - 0.9)]^{1/1.004} = 954.6 \mu\text{M}$



Since in the mixture  $I_1:I_2 = 190:1$ ,  
 then,  $(D_{90})_{1,2}$  can be dissected into:  
 $(D)_1 = 954.6 \times [190/(190 + 1)] = 949.6 \mu\text{M}$   
 $(D)_2 = 954.6 \times [1/(190 + 1)] = 4.998 \mu\text{M}$

$$\text{therefore, } (CI)_{90} = \frac{949.6 \mu\text{M}}{1511 \mu\text{M}} + \frac{4.998 \mu\text{M}}{13.62 \mu\text{M}} = 0.9955.$$

Since value of  $(CI)_{90}$  is close to 1, an additive effect of ADP-ribose and ADP at  $f_a = 0.9$  is indicated.

A computer program for automated calculation of  $m$ ,  $D_m$ ,  $D_1$ ,  $r$ , and  $CI$  at different  $f_a$  values has been developed.

#### Example 2. Inhibition of Alcohol Dehydrogenase by Two Mutually Non-Exclusive Inhibitors

Yonetani and Theorell (55) also studied the inhibition of horse liver alcohol dehydrogenase by the two competitive, mutually nonexclusive inhibitors: *o*-phenanthroline ( $I_1$ ) and ADP ( $I_2$ ). The fractional velocity ( $f_a$ ) values retrieved from the original plot are given in ref. 59, and are presented in the form of a median-effect plot, i.e.,  $\log [(f_a)^{-1} - 1]$  with respect to  $\log (I)$  (Fig. 4). *o*-Phenanthroline gives  $m = 1.303$ ,  $I_{50} = 36.81 \mu\text{M}$  and  $r = 0.9982$ ; and ADP gives  $m = 1.187$ ,  $I_{50} = 1.656 \mu\text{M}$  and  $r = 0.9842$ . These data again show that both inhibitors follow first order kinetics (i.e.,  $m \approx 1$ ). However, when the data for

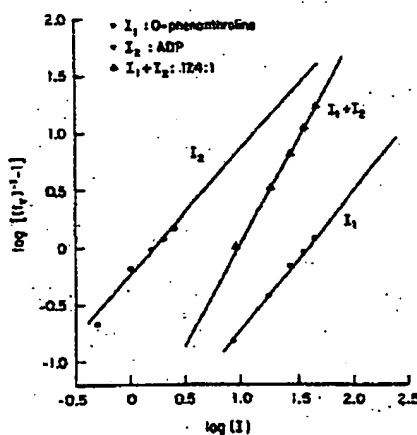


FIG. 4. The median-effect plot of experimental data of Yonetani and Theorell (55) for the inhibition of horse liver alcohol dehydrogenase by two mutually nonexclusive inhibitors.  $I_1$  is *o*-phenanthroline,  $I_2$  is ADP, and  $I_1 + I_2$  is a mixture of *o*-phenanthroline and ADP (molar ratio 174:1). The abscissa represents  $\log (I)_1$  ( $\odot$ ),  $\log (I)_2$  ( $\circ$ ), or  $\log [(I)_1 + (I)_2]$  ( $\Delta$ ) (from Chou and Talalay (59)).

the mixture of *o*-phenanthroline and ADP (constant molar ratio 17.4:1) are plotted in the same manner, a very different result is obtained:  $m_{1,2} = 1.742$  (apparent),  $(I_{50})_{1,2} = 9.116 \mu\text{M}$  and  $r = 0.9999$ . The dramatic increase in the slope of the plot for the mixture (in comparison to each of its components), clearly indicates that *o*-phenanthroline and ADP are mutually nonexclusive inhibitors.

The combination indices at various  $f_a$  levels are given in Figure 5. The results indicate that there is a moderate antagonism at low  $f_a$  values and a marked synergism at high  $f_a$  values.

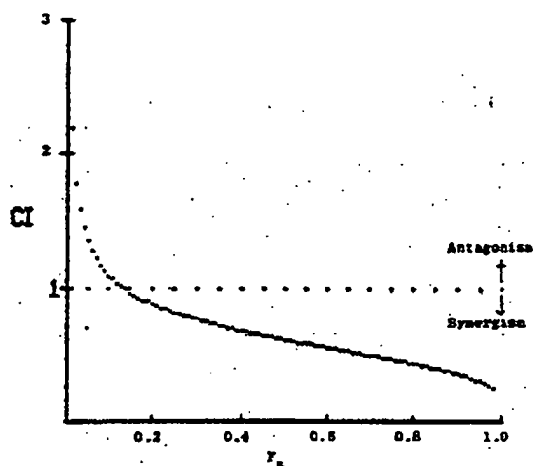


FIG. 5. Computer-generated graphical presentation of the combination index (CI) with respect to fraction affected ( $f_a$ ) for the inhibition of horse liver alcohol dehydrogenase by a mixture of *o*-phenanthroline and ADP (molar ratio 17.4:1). The method of analysis is the same as that described in the legend to Fig. 3, except that equation 10 (mutually non-exclusive) is used.

**Example 3. Inhibition of the Incorporation of Deoxyuridine into the DNA of L1210 Leukemia Cells by Methotrexate (MTX) and 1- $\beta$ -D-Arabinofuransylcytosine (ara-C)**

Murine L1210 leukemia cells were incubated in the presence of a range of concentrations of MTX (0.1–6.4  $\mu\text{M}$ ), of ara-C (0.0782–5.0  $\mu\text{M}$ ), or a constant molar ratio mixture of MTX and ara-C (1:0.782), and the incorporation of deoxyuridine into DNA was then determined. The fractional inhibitions ( $f_a$ ) of dUrd incorporation are shown in Table 1. Analysis of the results by the median effect plot (Fig. 6) gave the following parameters: for MTX,  $m = 1.091$ ,  $D_m = 2.554 \mu\text{M}$ ,  $r = 0.9842$ ; for ara-C,  $m = 1.0850$ ,  $D_m = 0.06245 \mu\text{M}$ , and

9.6  $\mu\text{M}$ 

9955.

effect of ADP-ribose and

on of  $m$ ,  $D_m$ ,  $D_a$ ,  $r$ , and CI at

**Two Mutually Non-**

inhibition of horse liver alcohol  
nonexclusive inhibitors: *o*-  
velocity ( $f_a$ ) values retrieved  
presented in the form of a  
plot to  $\log(I)$  (Fig. 4). *o*-  
 $r = 0.9982$ , and ADP gives  
data again show that both  
however, when the data for

2.5

and Theorell (55) for the  
nonexclusive inhibitors.  $I_1$  is *o*-  
phenanthroline and ADP (molar ratio  
 $I_1 + I_2$ ) ( $\Delta$ ) [from Chou and

TABLE 1. INHIBITION OF [6-<sup>3</sup>H]DEOXYURIDINE (dUrd) INCORPORATION INTO DNA IN L1210 LEUKEMIA CELLS BY METHOTREXATE (MTX) AND 1- $\beta$ -D-ARABINOFURANOSYLCYTOSINE (ARA-C), ALONE AND IN COMBINATION

MTX $\mu$ M	Fractional inhibition ( $\xi$ ) at [ara-C] of							
	0	0.782 $\mu$ M	0.156 $\mu$ M	0.313 $\mu$ M	0.625 $\mu$ M	1.25 $\mu$ M	2.5 $\mu$ M	5.0 $\mu$ M
0	0	0.582	0.715	0.860	0.926	0.955	0.980	0.993
0.1	0.0348	0.405						
0.2	ND*		0.587					
0.4	ND			0.775				
0.8	0.140				0.878			
1.6	0.415					0.943		
3.2	0.573						0.970	
6.4	0.755							ND

\*Result not used because of large variation between duplicates.

L1210 murine leukemia cells ( $8 \times 10^4$  cells) were incubated in Eagle's basal medium (20) in the presence and absence of various concentrations of MTX and ara-C and their mixture (molar ratio, 1:0.782) at 37°C for 20 min and then incubated with 0.5  $\mu$ M (1  $\mu$ Ci) of [6-<sup>3</sup>H]dUrd, at 37°C for 30 min. Fractional inhibition ( $\xi$  or  $\xi_c$ ) of [6-<sup>3</sup>H]dUrd incorporation into perchloric acid-insoluble DNA fraction was then measured as previously described (20). All measurements were made in duplicate.

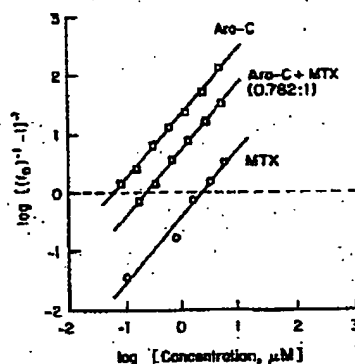


FIG. 6. Median-effect plot showing the inhibition of [6-<sup>3</sup>H]dUrd incorporation into DNA of L1210 murine leukemia cells by methotrexate (MTX), (O); arabinofuranosylcytosine (ara-C), (X); or their mixture (1:0.782), (+). Data from Table 1 have been used.

$r = 0.9995$ . For the combination of MTX and ara-C (1:0.782), the parameters were:  $m = 1.1296$ ,  $D_m = 0.2496 \mu$ M, and  $r = 0.9995$ . The combination index (Fig. 7) shows a moderate antagonism between the two drugs at all values of fractional inhibition.

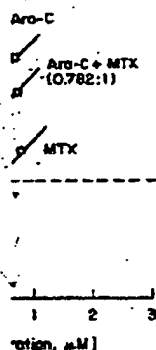
# DINE (dUrd) INCORPORATION INTO METHOTREXATE (MTX) AND 1- $\beta$ -D- ARABINOFURANOSYLCYTOSINE (ara-C), ALONE AND IN COMBINATION

fraction affected ( $f_a$ ) of

0.625 $\mu$ M	1.25 $\mu$ M	2.5 $\mu$ M	5.0 $\mu$ M
0.926	0.955	0.980	0.993
0.878	0.943	0.970	ND

mean duplicates.

incubated in Eagle's basal medium (20) in the presence of MTX and ara-C and their mixture (molar ratio 1:0.782) of [ $^3$ H]dUrd, at 37°C. [ $^3$ H]dUrd incorporation into perchloric acid-insoluble fractions was determined. All measurements were



of [ $^3$ H]dUrd incorporation into DNA of L1210 leukemia cells (O); arabinofuranosylcytosine (ara-C), (X); from Table 1 have been used.

and ara-C (1:0.782), the parameters  $r = 0.9995$ . The combination index between the two drugs at all values of

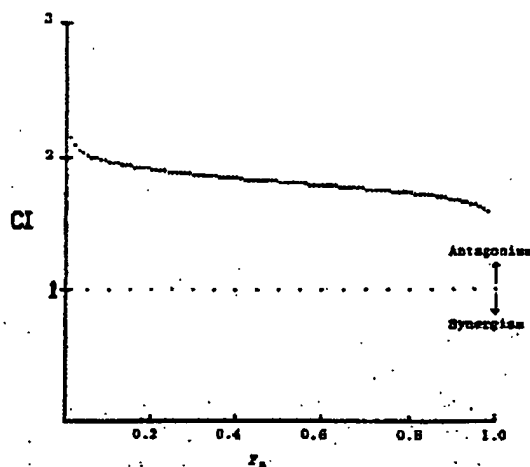


FIG. 7. Computer-generated graphical presentation of the drug combination index (CI) with respect to fraction affected ( $f_a$ ) for the inhibitory effect of a mixture of methotrexate (MTX) and arabinofuranosylcytosine (ara-C) (molar ratio, 1:0.782) on the incorporation of [ $^3$ H]dUrd into DNA of L1210 murine leukemia cells. The data from Table 1 and the parameters obtained from Fig. 6 have been used for this plot on the assumption that the drugs act in a mutually exclusive manner (equation 9).

## Example 4. Inhibition of the Incorporation of Deoxyuridine into the DNA of L1210 Leukemia Cells by Hydroxyurea (HU) and 5-Fluorouracil (5-FU)

Murine L1210 leukemia cells were incubated in the presence of a range of concentrations of hydroxyurea (50–3,200  $\mu$ M), or 5-fluorouracil (4.0–256  $\mu$ M), and of a constant molar ratio mixture of hydroxyurea and 5-fluorouracil (12.5:1), and the incorporation of deoxyuridine (dUrd) into DNA was then determined. The fractional inhibitions ( $f_a$ ) of dUrd incorporation are shown in Table 2. Analysis of the results by the median effect plot (Fig. 8) gave the following parameters: for hydroxyurea,  $m = 1.196$ ,  $D_m = 34.09 \mu$ M, and  $r = 0.9908$ ; for 5-fluorouracil,  $m = 1.187$ ,  $D_m = 8.039 \mu$ M, and  $r = 0.9978$ ; and for the mixture of hydroxyurea and 5-fluorouracil (12.5:1),  $m = 1.407$ ,  $D_m = 225.8 \mu$ M, and  $r = 0.9776$ . It is immediately apparent from Figure 8 that the effects of hydroxyurea and 5-fluorouracil are markedly antagonistic since the median-effect plot for the mixture lies to the right of both parent compounds. The degree of this antagonism falls as the level of inhibition increases, i.e. as  $f_a$  increases (Table 3). The reasons for this marked antagonism are obscure, but may be of practical importance.

TABLE 2. INHIBITION OF [6-<sup>3</sup>H]DEOXYURIDINE INCORPORATION INTO DNA IN L1210 LEUKEMIA CELLS BY HYDROXYUREA (HU), AND 5-FLUOROURACIL (5-FU), ALONE AND IN COMBINATION

HU μM	Fractional inhibition (f <sub>o</sub> ) at [5-FU] of							
	0	4 μM	8 μM	16 μM	32 μM	64 μM	128 μM	256 μM
0	0	0.348	0.475	0.661	0.827	0.923	0.966	0.985
50	0.605	0.208						
100	0.741		0.168					
200	0.889			0.345				
400	0.962				0.747			
800	0.984					0.885		
1,600	0.990						0.957	
3,200	0.994							0.977

L1210 murine leukemia cells ( $4.5 \times 10^6$ ) were incubated as described in the legend to Table 1, except that the incubation period with drugs prior to the addition of [6-<sup>3</sup>H]dUrd was 40 min. The results are analyzed in Fig. 8 and Table 3.

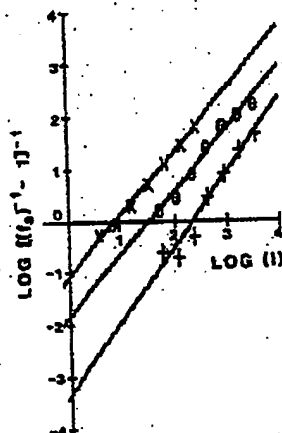


FIG. 8. Computer-generated median effect plot showing the inhibition of the incorporation of [6-<sup>3</sup>H]dUrd into DNA of L1210 murine leukemia cells by hydroxyurea (HU), (o); 5-fluorouracil (5-FU), (x), or their mixture (12.5:1), (+). The data given in Table 2 have been used. The parameters ( $m$ ,  $D_{50}$  and  $r$ ) can be obtained automatically.

#### Example 5. The Lethal Effects of Two Insecticides on Houseflies

Nearly 50 years ago Le Pelley and Sullivan (60) reported very careful dose-effect data for the lethality of rotenone, pyrethrins, and a mixture of these insecticides on houseflies. Adult houseflies were sprayed with alcoholic solutions of rotenone, pyrethrins, and a mixture of the two insecticides in a

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COMBINATION

[H] of		
	128 μM	256 μM
0.966	0.966	0.985
0.957	0.957	0.977

used in the legend to Table 1,  
[6-<sup>3</sup>H]dUrd was 40 min.

TABLE 3. CALCULATED VALUES FOR THE COMBINATION INDEX AS A  
FUNCTION OF FRACTIONAL INHIBITION ( $F_x$ ) OF THE INCORPORATION OF  
[6-<sup>3</sup>H]DEOXYURIDINE INTO DNA OF L1210 LEUKEMIA CELLS BY A MIXTURE  
OF HYDROXYUREA AND 5-FLUOROURACIL (MOLAR RATIO 12.5:1)

Fractional inhibition ( $F_x$ )	Combination index (CI)	Diagnosis of combined effect
0.05	11.95	Antagonism
0.10	10.86	Antagonism
0.20	9.80	Antagonism
0.30	9.15	Antagonism
0.40	8.65	Antagonism
0.50	8.21	Antagonism
0.60	7.80	Antagonism
0.70	7.38	Antagonism
0.80	6.89	Antagonism
0.90	6.21	Antagonism
0.95	5.61	Antagonism

The combination index was calculated on the assumption that the two drugs are mutually exclusive (Eqn. 9). The combination index was generated by computer on the basis of parameters obtained from the median effect plot (Fig. 8).

ratio by weight of 1:5. One thousand flies were used for each dose. The data are of historical interest since four different laboratories have attempted to answer the question whether there is synergism among these insecticides. The numerical results were retrieved by Finney (9) from the diagrams contained in the original paper (60).

These quantal data were equally suitable for analysis by the median effect principles as were the earlier examples in which graded responses were analyzed. We have recently provided a preliminary analysis of these results (13). The parameters of the median effect equation are shown in Table 4.

The dose-effect relationships for rotenone, pyrethrins and their mixture are clearly sigmoidal (Fig. 9A) with slopes ( $m$  values) ranging from 2.52 to 2.75 (Fig. 9B and Table 4). The regression coefficients ( $r$ ) are greater than 0.993, indicating that the applicability of the method to the data is excellent. The median-lethal doses ( $LD_{50}$ 's) for rotenone, pyrethrins and their mixture (1:5) as calculated from the median-effect plot are 0.157, 0.916 and 0.450 mg/cc, respectively (Fig. 9B). These values are in close agreement with those obtained from probit analysis by Finney (9) who obtained 0.156, 0.918 and 0.455 mg/cc, respectively.

The original authors interpreted the results as indicating no striking antagonistic or synergistic effect of the mixture. Richardson used a predictive method for the mixture equivalent to the similar action law (9) and asserted that there was pronounced synergism. Bliss supported Richardson's conclusion, and Finney, after a new analysis of data, also agreed that there

The incorporation of [6-<sup>3</sup>H]dUrd and 5-fluorouracil (5-FU) was used. The parameters

flies

reported very careful  
and a mixture of  
with alcoholic  
two insecticides in a

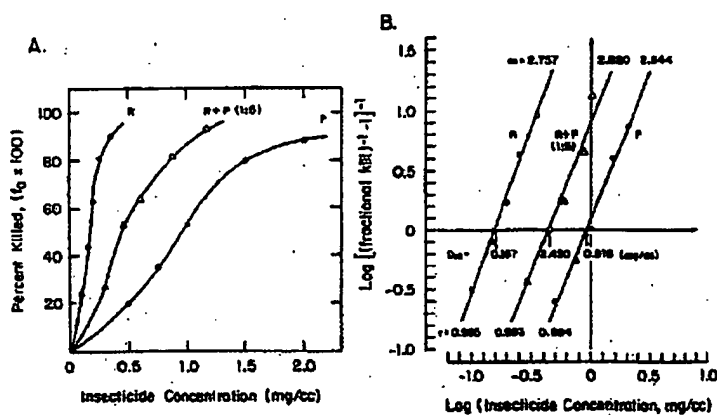


FIG. 9. Lethality of rotenone and pyrethrins to houseflies. Experimental data of LePelley and Sullivan (60), retrieved in ref. 13, were plotted for rotenone (R), pyrethrins (P), and their mixture (1:5, by weight) (R + P), on an arithmetic scale (A), and according to the median effect plot (B).

TABLE 4. TOXICITY OF ROTENONE AND PYRETHRINS TO HOUSEFLIES (60)

Insecticide	Parameters of median effect equation			
	m	$D_{50}$ (mg/cc)	Intercept	r
Rotenone	2.757 ( $\pm 0.157$ )	0.1571	2.216	0.9952
Pyrethrins	2.528 ( $\pm 0.158$ )	0.0907	0.0964	0.9960
Rotenone-Pyrethrins mixture (1:5)	2.519 ( $\pm 0.162$ )	0.4497	0.8743	0.9938

The m values ( $\pm$  S.E.) are the slopes of plots of  $\log [(1/(1-L)) - 1]^{-1}$  with respect to  $\log (D)$ , and were obtained by simple regression analysis on a programmable electronic calculator which also gives the y-intercepts and the linear regression coefficients (r). The median effect concentration is given by  $\text{antilog} (-y\text{-intercept}/m)$ . These results may also be obtained by the use of a computer program developed for this purpose (37).

was evidence of synergism (9). The present paper uses a new method and shows that rotenone and pyrethrins are indeed somewhat synergistic, as shown quantitatively in Figure 10.

#### SUMMARY

A generalized method for analyzing the effects of multiple drugs and for determining summation, synergism and antagonism has been proposed. The

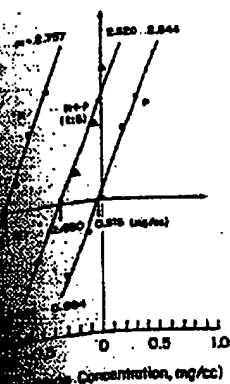


FIG. 9. Median effect plot (B) showing the relationship between log concentration and log effect for a mixture of rotenone and pyrethrins (ratio of 1:5, by weight). The data of LeFollet and Talalay (1964) were used.

#### HOUSEFLIES (60)

Effect equation	r
$E = \frac{1}{1 + 10^{(D - 0.216)}}$	0.9952
$E = \frac{1}{1 + 10^{(D - 0.0964)}}$	0.9960
$E = \frac{1}{1 + 10^{(D - 0.0743)}}$	0.9938

Effect to log (D), and were used to calculate the r value which also gives the concentration is given by a computer program

new method and that synergistic, as

the drugs and for the proposed. The

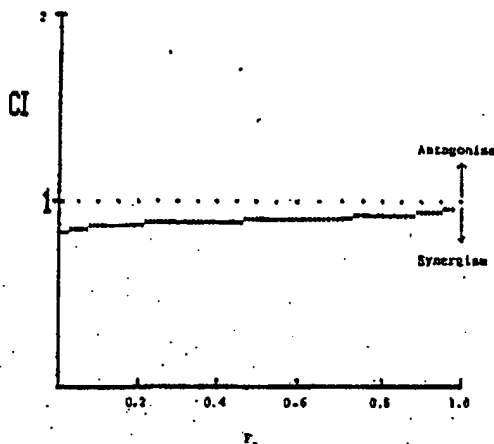


FIG. 10. Computer-generated graphical presentation of the combination index (CI) with respect to fraction affected ( $f_a$ ) for the lethality to houseflies of a mixture of rotenone and pyrethrins (ratio of 1:5, by weight). The data of ref. 13 (Fig. 9) and the method of calculation described in the legend to Fig. 3 were used.

derived, generalized equations are based on kinetic principles. The method is relatively simple and is not limited by 1) whether the dose-effect relationships are hyperbolic or sigmoidal, 2) whether the effects of the drugs are mutually exclusive or nonexclusive, 3) whether the ligand interactions are competitive, noncompetitive or uncompetitive, 4) whether the drugs are agonists or antagonists, or 5) the number of drugs involved.

The equations for the two most widely used methods for analyzing synergism, antagonism and summation of effects of multiple drugs, the isobologram and fractional product concepts, have been derived and been shown to have limitations in their applications. These two methods cannot be used indiscriminately. The equations underlying these two methods can be derived from a more generalized equation previously developed by us (59). It can be shown that the isobologram is valid only for drugs whose effects are mutually exclusive, whereas the fractional product method is valid only for mutually nonexclusive drugs which have hyperbolic dose-effect curves. Furthermore, in the isobol method, it is laborious to find proper combinations of drugs that would produce an iso-effective curve, and the fractional product method tends to give indication of synergism, since it underestimates the summation of the effect of mutually nonexclusive drugs that have sigmoidal dose-effect curves. The method described herein is devoid of these deficiencies and limitations.



The simplified experimental design proposed for multiple drug-effect analysis has the following advantages: 1) It provides a simple diagnostic plot (i.e., the median-effect plot) for evaluating the applicability of the data, and provides parameters that can be directly used to obtain a general equation for the dose-effect relation; 2) the analysis which involves logarithmic conversion and linear regression can be readily carried out with a simple programmable electronic calculator and does not require special graph paper or tables; and 3) the simplicity of the equation allows flexibility of application and the use of a minimum number of data points. This method has been used to analyze experimental data obtained from enzymatic, cellular and animal systems.

#### ACKNOWLEDGEMENTS

The authors wish to thank Joseph H. I. Chou of The Lawrenceville School, Lawrenceville, New Jersey, for developing the computer programs. These studies were supported by Grants from the National Institutes of Health (CA 27569, CA 18856 and AM 07422) and from the American Cancer Society (SIG-3).

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proposed for multiple drug-effect. It provides a simple diagnostic plot, the applicability of the data, and is used to obtain a general equation for which involves logarithmic conversion and is worked out with a simple programmable special graph paper or tables; and 3) simplicity of application and the use of a method has been used to analyze static, cellular and animal systems.

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APPENDIX

DERIVATION OF FRACTIONAL PRODUCT EQUATION AND ISOBOLOGRAM EQUATION. COMPARISON OF APPLICABILITY AND LIMITATIONS OF THESE METHODS

Derivation of Fractional Product Equation

Many investigators have assumed that the summation of the effects of two inhibitors can be expressed by the product of the fractional activities, i.e.  $(f_i)_{1,2} = (f_i)_1 \times (f_i)_2$ , as formalized by Webb (42) on intuitive grounds or assuming independence of inhibitor action.

Since  $f_i = 1 - f_i$ , equation 3 can be simplified (surprisingly) to:

$$\text{or } [1 - (f_i)_{1,2}] = [1 - (f_i)_1] [1 - (f_i)_2] \quad (11)$$

It is, therefore, clear that the fractional product equation describes only mutually nonexclusive first-order behavior.

Limitations of Fractional Product Method

The widely used fractional product concept of Webb (see equation 11) has serious limitations when applied to the analysis of the effect of multiple drugs. This method does not take into consideration the possible sigmoidicity of the dose-effect curve and the case where the drugs are mutually exclusive. The sample calculations shown in Table 5 illustrate the fact that the fractional product method is valid with respect to the present method only when the following conditions are satisfied: 1)  $D_1$  and  $D_2$  are mutually nonexclusive drugs, and 2) the dose-effect relationships of both  $D_1$  and  $D_2$  follow Michaelis-Menten-type hyperbola (i.e.,  $m = 1$ ).

As shown in Table 5, when the dose-effect curve is sigmoidal (e.g.,  $m > 1$ ), the fractional product method grossly underestimates the combined effect for mutually nonexclusive drugs and, in some cases, mutually exclusive drugs and, thus, may lead to false claims of synergism. In the case where the dose-effect curve is inversely sigmoidal (i.e.,  $m < 1$ ), the fractional product method will overestimate the combined effect, and thus lead to false claims of antagonism. However, the inverse sigmoidicity which is equivalent to negative cooperativity in enzyme systems is a relatively rare phenomenon. The aforementioned underestimation or overestimation of combined effects is particularly prominent at low  $f_i$  values. Table 5 also indicates that mutually nonexclusive drugs produce greater combined effects than mutually exclusive drugs at any level of sigmoidicity of the dose-effect curves.

TABLE 5. COMPARISON OF PREDICTIONS OF COMBINED EFFECTS OF MUTUALLY NONEXCLUSIVE AND EXCLUSIVE DRUGS ACCORDING TO THE FRACTIONAL PRODUCT (WEBB) METHOD AND THE PRESENT METHOD

Sigmoidicity of dose-effect curve for $D_1$ and $D_2$ (m value)	Effect level compared (when $(E_0)_1$ and $(E_0)_2$ are equal)	Combined effect, $(E_{0.5})_2$ predicted by		
		Fractional product method*	Present method for mutually nonexclusive drugs†	Present method for mutually exclusive drugs‡
0.5‡	0.05	0.0975	0.06932	0.06928
	0.1	0.19	0.1362	0.1358
	0.3	0.51	0.3878	0.3774
	0.5	0.75	0.6340	0.5858
	0.7	0.91	0.8643	0.7674
	0.9	0.99	0.9880	0.9272
1	0.05	0.0975	0.0975	0.0952
	0.1	0.19	0.19	0.1818
	0.3	0.51	0.51	0.4615
	0.5	0.75	0.75	0.6667
	0.7	0.91	0.91	0.8235
	0.9	0.99	0.99	0.9474
2	0.05	0.0975	0.2074	0.1739
	0.1	0.19	0.3769	0.3077
	0.3	0.51	0.7513	0.6316
	0.5	0.75	0.9	0.8
	0.7	0.91	0.9667	0.9032
	0.9	0.99	0.9956	0.9730
3	0.05	0.0975	0.4134	0.2963
	0.1	0.19	0.6291	0.4706
	0.3	0.51	0.8995	0.7742
	0.5	0.75	0.9643	0.8889
	0.7	0.91	0.9885	0.9492
	0.9	0.99	0.9984	0.9863

\*The fractional product concept depicted by equation 3 or 11. This method does not take into consideration the sigmoidicity of dose-effect curves and the exclusivity of effects of drugs.

†The median-effect principle of multiple drugs depicted by equation 5.

‡The median-effect principle of multiple drugs depicted by equation 4.

§Inverse sigmoidicity ( $m < 1$ ) is equivalent to negative cooperativity and is a relatively rare phenomenon.

#### Derivation of $ED_{50}$ Isobol Equations

The isobol or isobologram for representing the equi-effective graph of the combination of two drugs at their various doses was previously proposed by Loewe (40, 41) for analyzing synergism, summation and antagonism of effects.

It may be seen from equations 2-5 that, for the special case, when  $(E_{0.5})_2 = 0.5$ , the above relationships are all equal to 1 and hence the magnitude of the values of  $m$  (i.e. the sigmoidicity of the dose-effect curve) for the drugs is

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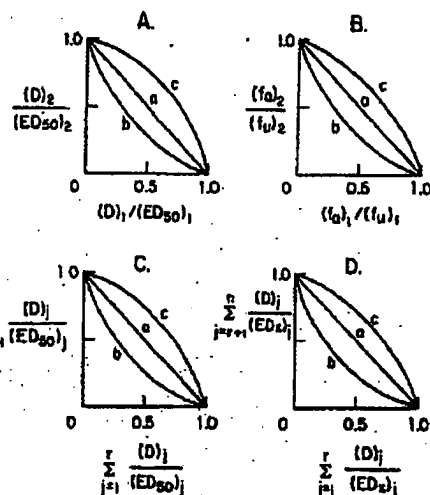


FIG. 11. Theoretical isobols for two or more drugs whose effects are mutually exclusive. A and B,  $ED_{50}$ -isobols for two drugs. C,  $ED_{50}$ -isobol for  $n$  drugs, and D,  $ED_{50}$ -isobol for  $n$  drugs. The straight lines, a, in isobolograms A, B, and C are based on equations 6, 12, and 20, respectively, and the straight line, a, in D is based on equation 21. In all cases, the additive iso-effective plot gives a straight line. If a concave upward curve or data point located below the straight line occurs (e.g., for A, curve b,  $[(D_1)/(ED_{50}1)] + [(D_2)/(ED_{50}2)] < 1$ ), then synergism is indicated. If a concave downward curve or data point located above the straight line occurs (e.g., for A, curve c,  $[(D_1)/(ED_{50}1)] + [(D_2)/(ED_{50}2)] > 1$ ), then antagonism is indicated. For mutually nonexclusive drugs, the isobologram cannot be used, since the equation contains a third term (see equations 3, 5 and 7). The limitations in using the isobologram are discussed in the Appendix.

irrelevant. For instance, equation 2 or 4 (for mutually exclusive drugs) become the equation which describes Loewe's isobologram (Fig. 11A):

$$\frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} = 1 \quad (6)$$

which can now be extended to (Fig. 11B):

$$\frac{(f_a)_1}{(f_a)_1} + \frac{(f_a)_2}{(f_a)_2} = 1 \quad (12)$$

where the two terms on the left can be assigned to the x and y axes, and equation 3 (for mutually nonexclusive drugs) becomes:

$$\frac{(f_a)_1}{(f_u)_1} + \frac{(f_a)_2}{(f_u)_2} + \frac{(f_a)_1 (f_a)_2}{(f_u)_1 (f_u)_2} = 1 \quad (13)$$

or

$$\frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} + \frac{(D)_1 (D)_2}{(ED_{50})_1 (ED_{50})_2} = 1 \quad (7)$$

Since there is an additional term in equation 7 or 13 when compared with equation 6 or 12, Loewe's isobologram is valid for mutually exclusive drugs but is not valid for mutually nonexclusive drugs.

#### Derivation of Generalized Isobol Equation

For the  $ED_x$ -isobol for any degree of effect, the general equation for summation for mutually exclusive agents is:

$$\frac{(D)_1}{(ED_x)_1} + \frac{(D)_2}{(ED_x)_2} = 1 \quad (8)$$

where  $(D)_1$  and  $(D)_2$  are the doses of drug 1 and drug 2 alone, respectively, that give  $x$  percent affected, and  $(D)_1$  and  $(D)_2$  (in the numerators) in combination give  $x$  percent affected [i.e.,  $(D_x)_{1,2} = (D)_1 + (D)_2$ ].

Equation 8 is valid irrespective of the  $m$  value and irrespective of the dose ratio of  $D_1$  and  $D_2$  that, in combination, would produce an  $f_a$  value of  $x$  percent. Equation 8 can be derived as follows:

- If we assume:
- 1)  $D_1$  and  $D_2$  are mutually exclusive drugs
  - 2)  $D_1$  and  $D_2$  both follow  $m^{\text{th}}$  order kinetics
  - 3) Dose ratio of  $D_1$  and  $D_2$  in combination is 1:a (i.e.,  $D_2 = aD_1$ )
  - 4)  $D_1$  and  $D_2$  in combination affect a target system  $x$  percent which produces  $[(f_a)_x]_{1,2}$ .

From equation 4, for two drugs in combination, we obtain:

$$\left\{ \frac{[(f_a)_x]_{1,2}}{1 - [(f_a)_x]_{1,2}} \right\}^{\frac{1}{m}} = \frac{(D)_1}{(ED_{50})_1} + \frac{(D)_2}{(ED_{50})_2} = \frac{(D)_1}{(ED_{50})_1} + \frac{a(D)_1}{(ED_{50})_2}$$

$$= \frac{(D)_1 (ED_{50})_2 + a(D)_1 (ED_{50})_1}{(ED_{50})_1 (ED_{50})_2} \quad (14)$$

Let the term or

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$$\frac{(D)_1}{(ED_x)_1} + \frac{(D)_2}{(ED_x)_2}$$

Therefore, equ

Let the term on the left be represented by A (i.e.,  $A = \{[(f_x)_i]^{-1} - 1\}^{1/m}$ ). Then:

$$(D)_1 = \frac{A(ED_{50})_1 (ED_{50})_2}{a(ED_{50})_1 + (ED_{50})_2} \quad (15)$$

$$(D)_2 = \frac{aA(ED_{50})_1 (ED_{50})_2}{a(ED_{50})_1 + (ED_{50})_2} \quad (16)$$

When each drug alone affects a target system x percent (i.e.  $(ED_x)_1$  produces  $[(f_x)_1]$  and  $(ED_x)_2$  produces  $[(f_x)_2]$ , then, from equation 1, we obtain:

$$\left\{ \frac{[(f_x)_1]}{1 - [(f_x)_1]} \right\}^{\frac{1}{m}} = \frac{(ED_x)_1}{(ED_{50})_1} \quad (17)$$

and

$$\left\{ \frac{[(f_x)_2]}{1 - [(f_x)_2]} \right\}^{\frac{1}{m}} = \frac{(ED_x)_2}{(ED_{50})_2} \quad (18)$$

At an iso-effective condition,  $[(f_x)_1]$ ,  $[(f_x)_2]$  and  $[(f_x)_x]$  are all equal (i.e., equal to A, or  $\{[(f_x)_i]^{-1} - 1\}^{1/m}$ ). Thus,  $(ED_x)_1 = A(ED_{50})_1$  and  $(ED_x)_2 = A(ED_{50})_2$ , and thus:

$$\begin{aligned} \frac{(D)_1}{(ED_x)_1} + \frac{(D)_2}{(ED_x)_2} &= \frac{A(ED_{50})_1 (ED_{50})_2}{[a(ED_{50})_1 + (ED_{50})_2]} + \frac{aA(ED_{50})_1 (ED_{50})_2}{[a(ED_{50})_1 + (ED_{50})_2]} \\ &= \frac{(ED_{50})_2}{a(ED_{50})_1 + (ED_{50})_2} + \frac{a(ED_{50})_1}{a(ED_{50})_1 + (ED_{50})_2} \\ &= 1 \end{aligned}$$

Therefore, equation 8 is confirmed.



Therefore, irrespective of the magnitude of the  $m$  value and irrespective of the ratio of doses in the combination (a), equation 8 for the  $ED_{50}$ -isobologram is always valid for mutually exclusive drugs.  $(ED_{50})_1$  and  $(ED_{50})_2$  in the numerator of equations 17 and 18, or the denominator of equation 8 can be readily obtained from equation 1 or the median-effect plot.  $(ED_{50})_{1,2}$  that produces the iso-effect can also be obtained from equation 1.  $(ED_{50})_{1,2}$  can then be dissected into its components  $(D)_1$  and  $(D)_2$  [i.e.,  $(ED_{50})_{1,2} = (D)_1 + (D)_2$ ]. Since the ratio of  $(D)_1$  and  $(D)_2$  in the mixture is known at the outset of the experiments [e.g.,  $(D)_1/(D)_2 = P/Q$ ],  $(D)_1$  and  $(D)_2$  in the numerators of equation 8 can be obtained by  $(D)_1 = (ED_{50})_{1,2} \times P/(P+Q)$  and  $(D)_2 = (ED_{50})_{1,2} \times Q/(P+Q)$ .

Therefore, the CI procedures described above, as depicted in equations 9 and 10, eliminate the labor and the limitations of the old isobologram method.

The present study also allows additional new isobolograms to be formulated:

For mutually exclusive drugs, the isobol for the combination of  $n$  inhibitors (59) can be described by:

$$\sum_{j=1}^n \frac{(L)_j}{(L_0)} = 1 \quad (19)$$

for the  $ED_{50}$ -isobologram, or by (Fig. 11C)

$$\sum_{j=1}^n \frac{(D)_j}{(ED_{50})_j} = 1 \quad (20)$$

which can now be extended to:

$$\sum_{j=1}^n \frac{(D)_j}{(ED_{50})_j} = 1 \quad (21)$$

for a generalized isobologram (see Fig. 11D).

The  $n$  can be partitioned into any combination of two parts (e.g.,  $\sum_{j=1}^n = \sum_{j=1}^m + \sum_{j=m+1}^n$  where  $1 \leq m \leq n-1$ ,  $m$  and  $n$  are integers) which can be assigned to the two coordinates of the isobol. Thus, a three-dimensional isobologram of great complexity (41, 45) will not be necessary, and the three-dimensional presentation of effects (45, 48) can be extended to four or more drugs and yet use only two-dimensional graphs.

*Limitations of the Isobologram Method*

Loewe's isobologram (40, 41) is valid for equations 8 or 12 but not for equation 7 or 13. Thus, mutually nonexclusive inhibitors (which are in fact additive in equation 13) would appear to be slightly synergistic in Loewe's isobologram.

Aside from the limitation of the isobologram and its applicability only to mutually exclusive drugs, the major practical drawback of the isobol method is that it is laborious to find proper combinations of drugs that would produce an iso-effect point. In all cases, general equations (equations 4 and 5 or 9 and 10) can be more conveniently used than the isobolograms since the latter are merely special cases of equation 4.

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